

Hepatitis C Virus (HCV)-Induced Immunoglobulin Hypermutation Reduces the Affinity and Neutralizing Activities of Antibodies against HCV Envelope Protein^{▽†}

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Hepatitis C virus (HCV) often causes persistent infection despite the presence of neutralizing antibodies against the virus in the sera of hepatitis C patients. HCV infects both hepatocytes and B cells through the binding of its envelope glycoprotein E2 to CD81, the putative viral receptor. Previously, we have shown that E2-CD81 interaction induces hypermutation of heavy-chain immunoglobulin (V_H) in B cells. We hypothesize that if HCV infects antibody-producing B cells, the resultant hypermutation of V_H may lower the affinity and specificity of the HCV-specific antibodies, enabling HCV to escape from immune surveillance. To test this hypothesis, we infected human hybridoma clones producing either neutralizing or non-neutralizing anti-E2 or anti-E1 antibodies with a lymphotropic HCV (SB strain). All of the hybridoma clones, except for a neutralizing antibody-producing hybridoma, could be infected with HCV and support virus replication for at least 8 weeks after infection. The V_H sequences in the infected hybridomas had a significantly higher mutation frequency than those in the uninfected hybridomas, with mutations concentrating in complementarity-determining region 3. These mutations lowered the antibody affinity against the targeting protein and also lowered the virus-neutralizing activity of anti-E2 antibodies. Furthermore, antibody-mediated complement-dependent cytotoxicity with the antibodies secreted from the HCV-infected hybridomas was impaired. These results suggest that HCV infection could cause some anti-HCV-antibody-producing hybridoma B cells to make less-protective antibodies.

Hepatitis C virus (HCV) infection often persists despite the presence of robust host immune responses, leading to chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and B-lymphocyte proliferative disorders, including mixed cryoglobulinemia, a disorder characterized by oligoclonal proliferation of B cells, and B-cell lymphoma (36, 52, 55). The viral genome is a single-stranded, positive-sense RNA of 9.6 kb. The predicted structural components of the viral particles comprise the core protein and two heavily N-glycosylated envelope glycoproteins, E1 and E2 (20). Both E1 and E2 are type I transmembrane proteins, with N-terminal ectodomains and C-terminal hydrophobic anchors.

HCV modifies the B-cell receptor-associated signaling pathway by binding to B-cell surface molecules. HCV infects liver cells, B cells, and probably other cells through CD81 and other receptor candidates (6, 46, 49, 50). CD81 is part of the CD21/

CD19/CD81 complex that serves as a coreceptor for B-cell receptor (15, 35). Recombinant E2 protein or E1-E2 heterodimers bind to cells in a CD81-dependent manner (6, 38). HCV envelope protein also stimulates T cells to secrete IL-4 (35) by binding to CD81 through Lck (56, 64) and inhibits natural killer (NK) cells through engagement of CD81 (8). To produce high-affinity antibodies, B cells target a high rate of somatic hypermutation (SHM) to the immunoglobulin (Ig) variable-region genes that encode the antigen-binding sites. This mutational process requires transcription and is triggered by activation-induced cytidine deaminase (AID), which converts deoxycytidine to deoxyuridine (25, 40, 41). We have shown that HCV infection or E2-CD81 interaction induces double-stranded DNA breaks specifically in Ig heavy chain (V_H) and also expression of AID, leading to hypermutation of V_H in B cells (38, 39). Thus, if HCV infects antibody-producing B cells, it is expected to trigger V_H hypermutation, thereby altering the property of antibody produced by the infected B cells. These mutations will likely affect the binding affinity, neutralizing activity and even the antibody-mediated complement-dependent cytotoxicity (CDC). These effects will lower the antiviral activities of the humoral antibodies. To date, no global immune suppression has been reported during HCV infection. Nevertheless, selective CD4 helper T cells defects

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TABLE 1. Mutation frequencies of V_H of HCV-infected hybridomas^a

Cell type	UV-inactivated HCV	HCV	% Clones mutated ^b	No. of nucleotides mutated*	Mutation frequency ^c (mutation/bp), 10 ⁻⁴	% Amino acids mutated in CDR ^d
CBH2	—	—	17 (4/26)	4	5.8	50 (1/2)
	+	—	20 (5/25)	5	7.6	33 (1/3)
	—	+	19 (5/26)	5	7.3	33 (1/3)
CBH4	—	—	5 (2/22)	3	5.2	100 (1/1)
	+	—	16 (4/27)	5	7.0	33 (1/3)
	—	+	47 (12/28)	15	20.2 ($P < 0.001$)	50 (4/8)
CBH5	—	—	12 (4/34)	6	6.7	33 (1/3)
	+	—	16 (6/38)	8	8.0	50 (2/4)
	—	+	34 (15/44)	21	18.1 ($P < 0.003$)	63 (10/16)
CBH17	—	—	11 (4/36)	4	4.2	50 (1/2)
	+	—	14 (5/35)	7	7.6	67 (2/3)
	—	+	49 (19/39)	25	24.3 ($P < 0.0002$)	63 (12/19)

^a Hybridomas (mixed B-cell population) were harvested for DNA sequence analysis on day 14 postinfection. The V_H gene was cloned into the pCDN3.1 plasmid. Multiple plasmid clones were sequenced to determine mutation frequencies (26).

^b The numbers of PCR clones containing at least one mutation versus the numbers of PCR clones sequenced are shown in parentheses.

^c The mutation frequency was calculated as the number of mutations versus the total base pairs sequenced.

^d The number of PCR clones containing at least a mutation versus total number of PCR clones sequenced is expressed in parentheses.

have been reported in chronic HCV patients (4, 5, 59, 60). It is conceivable that certain subsets of B cells may also be defective during HCV infection. This scenario will help to explain why the presence of HCV-specific antibodies in patient sera fails to neutralize HCV and prevent HCV infection.

Increasing evidence has shown that HCV infects not only liver but also B cells (57). Furthermore, HCV infection of B cells has causal effects on the clinical presentation of HCV infection, including B-cell lymphoma, as antiviral therapy caused remission of B-cell tumors (23, 34, 70). We have previously isolated a preferentially lymphotropic HCV strain (SB strain) from a B-cell lymphoma (57). This B-cell line is monoclonal and produces IgM antibody against HCV NS3 protein (unpublished observation), indicating that antibody-producing B cells can be infected by HCV in vivo. Recent studies have identified the envelope protein of the SB virus as the basis for its preferential lymphotropism (K. Machida et al., unpublished observation). Thus, B-cell involvement may represent an important facet of HCV infection. We hypothesize that if the antibody-producing B cells are infected by HCV, the resultant V_H hypermutation will likely affect the antiviral properties of these antibodies. This possibility may represent a novel mechanism of viral escape from immunosurveillance.

MATERIALS AND METHODS

Viruses and cells. Raji cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 supplemented with 20% fetal bovine serum. SB cells were established from an HCV (genotype 2b)-infected non-Hodgkin's B-cell lymphoma; SB cells continuously produces infectious HCV virions in culture (57). To express E2 proteins on the cell surface, the recombinant vaccinia virus expressing E2 (H strain, amino acids [aa] 371 to 661)-CD4 (aa 374 to 435) and E2 (aa 371 to 717)-CD4 (aa 374 to 435) were used (7). Human hybridomas (CBH2, CBH4, CBH5, and CBH17) producing antibodies specific for HCV E2 protein were previously described (21, 28). These hybridomas were made from Epstein-Barr virus (EBV)-transformed human B cells fused to a mouse-human cell line. CBH2 and CBH5 produce HCV-neutralizing antibody, whereas CBH4 and CBH17 produce non-neutralizing antibody. H111 is a hybridoma producing anti-E1 antibody (29). The cells were cultured in IMDM (Invitrogen) with 20% fetal bovine serum. Limiting dilution of hybridoma cells to isolate single-cell clones was performed as described previously (57).

ELISA. The expression of the recombinant proteins was assessed by enzyme-linked immunosorbent assay (ELISA) on naturally occurring lectin *Galanthus nivalis* agglutinin (Sigma, St. Louis, MO) as previously described (21).

Baculovirus expression and purification of HCV E1 and E2 proteins. The E2 sequences from a genotype 1a isolate (strain H77) (68) and genotype 1b isolate (strain HC-J4) (69) without the C-terminal transmembrane domains but containing a His₆ tag at the C termini, were cloned into a baculovirus transfer vector (pBlueBacHis2; Invitrogen, Carlsbad, CA) as previously described (32, 37). Expression of recombinant E2 proteins using the recombinant baculovirus in insect cells was performed as described in the Bac-N-Blue baculovirus expression system (Invitrogen) as previously described (37). Detailed descriptions of the methods used here can be found in the supplemental material.

Antibody coating and E2 binding assays. The following purified monoclonal antibodies were used: anti-human IgM (Caltag, Burlingame, CA), anti-human IgG and anti-CD19 B4 (Beckman Coulter, Fullerton, CA), anti-CD21 (BD Biosciences/PharMingen) (14), anti-CD81 (JS-81; BD Biosciences), and anti-E2 (Biodesign International, Saco, ME). Ninety-six-well plates were coated with the antibodies as described previously (8).

Quantitation of HCV RNA by RT-PCR. HCV RNA was detected by using the procedure as described in the previous report (57). More details concerning the reverse transcription-PCR (RT-PCR) can be found in the supplemental material.

Cloning and sequencing. Genomic DNA from the various cell lines was extracted according to standard methods. PCR amplification was performed using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and the reported primers for V_H (from V_H framework 1 to constant region 1 of heavy-chain genes) (26). Both strands were sequenced to confirm the mutations. Even if base changes in infected and uninfected cells were the same, both mutations in infected and uninfected cells were counted. For detailed descriptions of methods used here and below, see the supplemental material.

Virus neutralization assay. A virus neutralization assay was performed by incubating the SB culture supernatant with antibodies secreted from HCV-infected or uninfected CBH hybridomas for 2 h and then using the supernatant to infect naive Raji cells. The production of HCV virions from SB cell culture, HCV infection, and determination of viral RNA titers have been described previously (57).

CDC assay. CDC assay was conducted as follows. Briefly, stocks of vTF7-3 (a recombinant vaccinia viruses expressing the T7 DNA-dependent RNA polymerase) and vaccinia virus recombinants expressing E2 (H strain)-CD4 chimeric proteins were used to infect Huh7 cells (7). At 12 h postinfection, the infected cells were plated onto 96-well plates. A CDC assay was performed with antibodies from the various hybridomas and was measured by using a [⁵¹Cr]chromium release assay in the presence of complement in serial dilutions of human serum (Calbiochem) as previously described (24).

Statistical analysis. Statistical analysis of the data in Table 1 and in Table S1

in the supplemental material was performed by χ^2 test. *P* values of <0.05 were considered statistically significant.

RESULTS

HCV infects B cells producing neutralizing or non-neutralizing anti-E2 antibodies. We first assessed whether HCV could infect human monoclonal hybridoma cells producing either neutralizing or non-neutralizing anti-E2 antibodies. A previously described lymphotropic HCV (SB strain) produced from a B-cell lymphoma cell line (57) was used. For a negative control, SB virus was irradiated with UV prior to infection. Infected cells were harvested at various days postinfection, and the intracellular HCV RNA titer was determined. The results showed that HCV RNA could be detected in three of four anti-E2 antibody-producing hybridoma clones (CBH4, -5, and -7); the viral RNA titer peaked at day 14 and decreased thereafter, but HCV RNA could still be detected even at day 56 postinfection, indicating the establishment of persistent infection (Fig. 1B). The CBH2 clone, which produces a neutralizing anti-E2 antibody, could not be infected. Interestingly, the other neutralizing antibody-producing hybridoma, CBH5, could be infected with HCV, although to a lower extent than CBH4 and CBH17, which produce non-neutralizing anti-E2 antibodies, suggesting that the expression of surface Ig did not block virus entry. The H-111 clone, which produces monoclonal antibody against E1 (29), was also infectible with HCV to the same extent as the CBH4 clone. Furthermore, Raji cells and R04, hybridoma, the latter of which produces an irrelevant antibody (against a cytomegalovirus protein) could also be infected. As a negative control, viral RNA could not be detected in the cells inoculated with UV-irradiated virus or mock-infected cells (data not shown). Immunofluorescence studies demonstrated that HCV nonstructural protein 3 (NS3) was detected in hybridomas after HCV infection (Fig. 1C). We have previously shown that AID expression is induced by HCV infection (39); semiquantitative RT-PCR of AID mRNA indeed showed that CBH5 and CBH17, but not CBH2, had increased levels of AID upon HCV infection (Fig. 1D). As a comparison, CD81 was not affected (Fig. 1D). These results indicate that B cells producing non-neutralizing or irrelevant antibody can be infected with the lymphotropic HCV, although some hybridomas producing neutralizing antibody could also be infected.

HCV infection induces hypermutation of *V_H* in hybridoma cells. Previously, we have shown that HCV infection induces hypermutation of *V_H* in B cells (38, 39). We therefore determined the sequences of *V_H* gene in multiple individual clones derived from each infected hybridoma to calculate the mutation frequencies of *V_H*. At 16 days postinfection, *V_H* gene from HCV-infected cells (CBH4, CBH5, and CBH17) showed a significantly higher number of clones that contain mutated *V_H* genes. The mutation frequencies were calculated to be 18.1×10^{-4} to 24.3×10^{-4} mutations/bp, which is nearly three- to fivefold higher than that in the hybridomas mock-infected or infected with UV-irradiated HCV (4.2×10^{-4} to 8.0×10^{-4} mutations/bp) (Table 1). In the cells resistant to HCV infection (CBH2), the mutation frequency was the same as that in the uninfected cells. Previously, we have shown that E2-CD81 binding in vitro is sufficient to induce *V_H* hypermutation (38); therefore, we also used recombinant E2 proteins (genotype 1a)

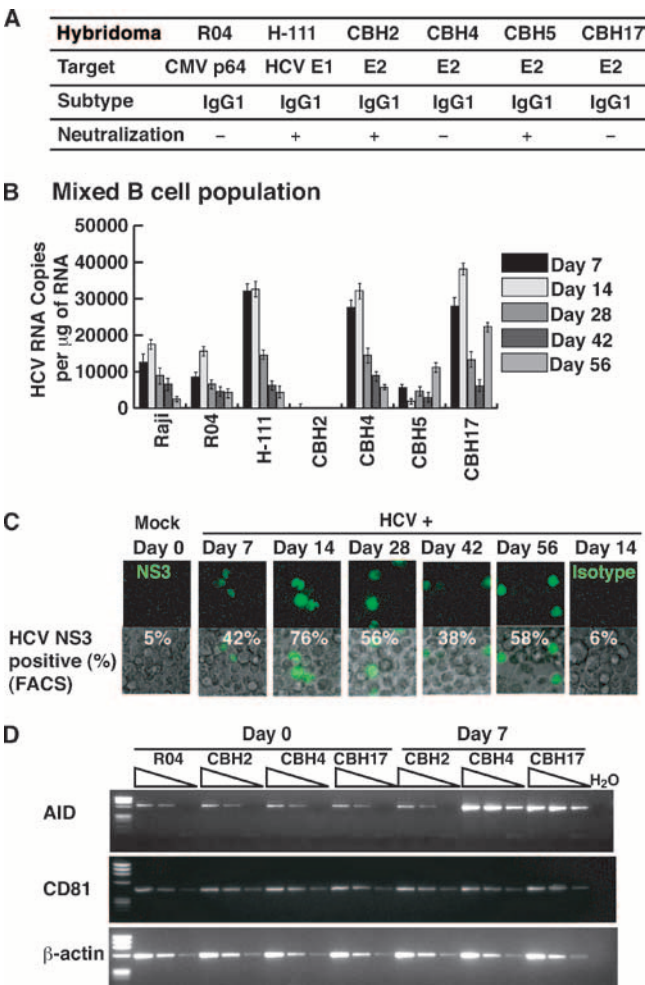


FIG. 1. (A) EBV-transformed human B cells fused to a mouse-human cell line (hybridoma) are listed with regard to protein targets, heavy-chain subtype, and neutralizing activity for the HCV 2b strain. (B) Infectivity of the hybridoma clones by SB strain of HCV. Total RNA was extracted from cells 7, 14, 28, 42, or 56 days postinfection. R04 is a hybridoma producing antibody against cytomegaloviral proteins. CBH4 and -17, hybridoma producing non-neutralizing anti-E2 antibody; CBH2 and -5, hybridomas producing neutralizing anti-E2 antibody. HCV RNA in the cells was quantified by real-time RT-PCR at various days after infection. The minimum detection limit was 80 copies. (C) Immunofluorescence of NS3 protein. (D) Semiquantitative RT-PCR for AID, CD81, and β -actin. The cDNA was serially diluted fivefold twice and used for PCR.

to stimulate hybridoma cells in vitro to compare with HCV infection in vivo (38). The results showed that the binding of recombinant E2 to hybridoma cells in vitro (38) induced an increased mutation frequency (8.6×10^{-4} to 14.5×10^{-4} mutations/bp), similar to those in the HCV-infected CBH clones (see Table S1 in the supplemental material). Furthermore, incubation with anti-IgG plus CD19/CD21 also yielded a comparable high mutation frequency. In contrast, E1 protein did not induce *V_H* hypermutation. These results combined indicated that HCV infection or E2 binding induces hypermutation of immunoglobulin in hybridoma cells that produce anti-E2 antibodies.

HCV infection induces amino acid changes of E2-specific antibodies. The observed enhancement of mutation frequency of V_H gene by HCV infection (38, 39) suggests that HCV infection may alter the properties of antibodies produced by these hybridoma clones. To test this possibility, each hybridoma clone was further subcloned, and V_H gene sequences in individual subclones were examined. Many of these clones exhibited nonsynonymous amino acid changes, most of which are clustered in CDR2 and CDR3 regions (Fig. 2). Some clones also included frameshift mutations or deletions. To determine if the decreases in binding of some subclones are not due to class switching, which is also caused by AID, Ig class was determined (Fig. 2B). We performed class switch frequency analysis in these hybridoma cells by PCR, showing that hybridoma cells did not undergo an Ig class switch despite the high AID level. These mutations are expected to affect the properties of the antibodies.

HCV-infected hybridomas produce low-affinity anti-E2 antibodies. To determine whether V_H mutations in the HCV-infected hybridomas altered the properties of antibodies, we first determined the relative antigen-binding activity of anti-E2 antibodies produced by these cells. ELISA was performed using purified recombinant E2 proteins and pools of antibodies from HCV-infected and uninfected hybridomas harvested at different days after infection. Each point was normalized with the same antibody concentration. We reported previously that HCV caused a significant (20%) apoptosis in B cells (57). Thus, the amount of antibody produced in the HCV-infected B cells was probably lower than that from the uninfected cells. Antibodies were purified and normalized for binding assay. The relative E2 binding, at equal antibody concentrations, of antibodies produced from the HCV-infected hybridomas (CBH4, -5, and -17) gradually declined to ca. 70% of the respective parental hybridomas by 8 weeks after infection (Fig. 3A). In contrast, the affinity of antibodies from mock-infected hybridoma or those infected with UV-irradiated virus remained unchanged. Furthermore, the antibodies from CBH2, which was not permissive to HCV infection, retained the original binding affinity even after 8 weeks. To determine whether the observed change of E2 binding was indeed due to the reduction of affinity from individual clones of hybridomas, limiting dilution of each infected hybridoma was performed to isolate single subclones. All of the subclones from uninfected hybridomas (HCV-) produced high-affinity antibodies against E2 protein; in contrast, some of the clones derived from HCV-infected hybridomas produced low-affinity anti-E2 antibodies (Fig. 3B, 3 of 17 CBH5 clones and 4 of 16 CBH17 clones). All of these clones have nonsynonymous mutations in the CDR3 region (Fig. 2) (clones 5-5, 5-10, 5-14, 17-4, 17-9, 17-12, and 17-19). Some of the weak binding antibodies (5-14, 17-9) were further studied by ELISA using different concentrations of antibodies. The level of binding activity of these antibodies was significantly lower than that from the uninfected hybridoma (clone 5-14 versus clone 5-35 and clone 17-9 versus clone 17-33) (Fig. 3C). These results indicated that HCV infection reduces the affinity of antibody for the targeting protein.

HCV-induced hypermutation inactivates virus-neutralizing activity. We next examined whether the mutations affected the virus-neutralizing activities of the antibodies. We incubated SB virus with CBH antibodies from different subclones. After in-

cubation, the infectivity of SB virus was then assayed on naive Raji cells. As shown in Fig. 4, when HCV was preincubated with various CBH5 antibodies (at 10 μ g/ml), no viral RNA was detected in Raji cells 14 days after infection; in contrast, viral RNA was detectable when the virus was pretreated with R04 isotype-matched antibody (the virus titer equivalent to that without antibody pretreatment), indicating that the virus was not neutralized. The pooled CBH5 antibodies reduced viral infectivity by more than 90%. Higher concentrations of CBH5 antibodies (up to 50 μ g/ml) did not further reduce viral infectivity (data not shown). This result suggests that CBH5 antibodies neutralized HCV infectivity; however, the fact that CBH5 cells could be infected suggests that the entry mechanism of SB virus is independent of surface Ig on B cells.

The antibody secreted from every subclone of the uninfected CBH5 hybridomas neutralized HCV infectivity. In contrast, the antibodies secreted from 3 of 17 subclones derived from HCV-infected CBH5 hybridoma lost neutralizing activity (Fig. 4). These three clones (i.e., clones 5-5, 5-10, and 5-14) carry mutations in the CDR3 region, and their antibodies have lower binding affinities for E2 protein (Fig. 2 and 3). As a control, CBH17 produced only non-neutralizing antibodies. These results indicate that HCV infection of the anti-HCV antibody-producing B cells resulted in the reduction of the virus neutralization activities of the antibodies.

HCV infection reduces CDC. Antibody binds to viral envelope protein expressed on the surface of HCV-infected cells and may recruit effector cells, such as NK cells or monocytes, by interacting with specific Fc receptors. As a result, the HCV-specific antibodies may induce CDC, leading to lysis of the infected cells. We therefore determined whether anti-E2 antibody (IgG) may mediate the CDC of E2-expressing Huh7 cells. To perform this assay, we first expressed E2-CD4 on the surface of Huh7 cells, as confirmed by fluorescence-activated cell sorting (FACS) analysis (Fig. 5A). E2-expressing Huh7 cells were then labeled with [51 Cr]chromate and incubated with antibodies from HCV-infected hybridomas in the presence of complement. As shown in Fig. 5B, antibodies from all uninfected CBH hybridomas mediated specific cell lysis to various extents (15 to 30% lysis). A control antibody R04 did not induce cell lysis. As a negative control, none of the antibodies lysed the target cells expressing E1 protein (data not shown). Those from HCV-infected hybridomas (CBH4, CBH5, and CBH17) had significantly lower CDC activities. In contrast, the CDC activity of the antibodies from the hybridomas infected with UV-irradiated HCV was similar to that from mock-infected cells. Similarly, the CDC activity of CBH2, which is resistant to HCV infection, was not affected by HCV infection. These results indicated that anti-E2 antibodies can induce specific CDC and that HCV infection reduced the CDC activities of these antibodies.

We further characterized the CDC of individual subclones from the infected hybridomas. As shown in Fig. 5C, antibodies with reduced binding affinities (clones 5-14, 17-9, and 17-19) (see Fig. 3) had very low CDC activities, but the strong binders (clones 5-35, 17-31, 17-33, 17-1, and 17-3) (see Fig. 3) had strong lysis activity (Fig. 5C). All of the low binders were from hybridomas infected with HCV. These results indicate that antibody affinity with E2 protein correlated with the CDC activity. These results strongly suggest that HCV-induced

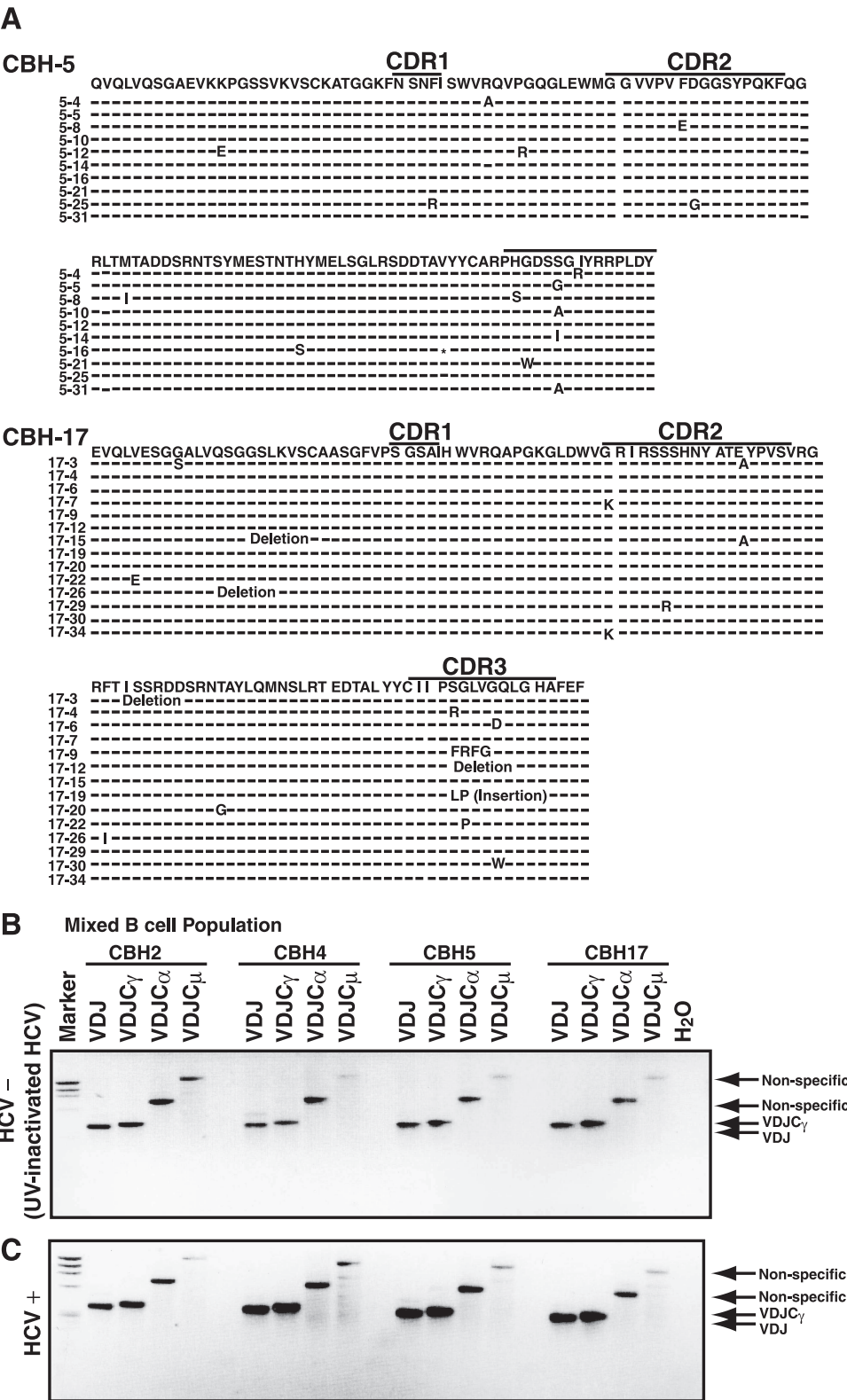


FIG. 2. (A) Amino acid sequences of V_H in the infected hybridomas. V_H genes were cloned from HCV-infected CBH5 and CBH17 hybridomas at 56 days postinfection, and multiple V_H clones were sequenced. Only clones containing nonsynonymous mutations are shown. (B) The subtype of heavy chain of Ig was determined by PCR analysis. PCR amplification of the VDJ region served as a positive control. HCV-infected hybridomas at day 14 postinfection were also tested for subtypes of the heavy chain of Ig.

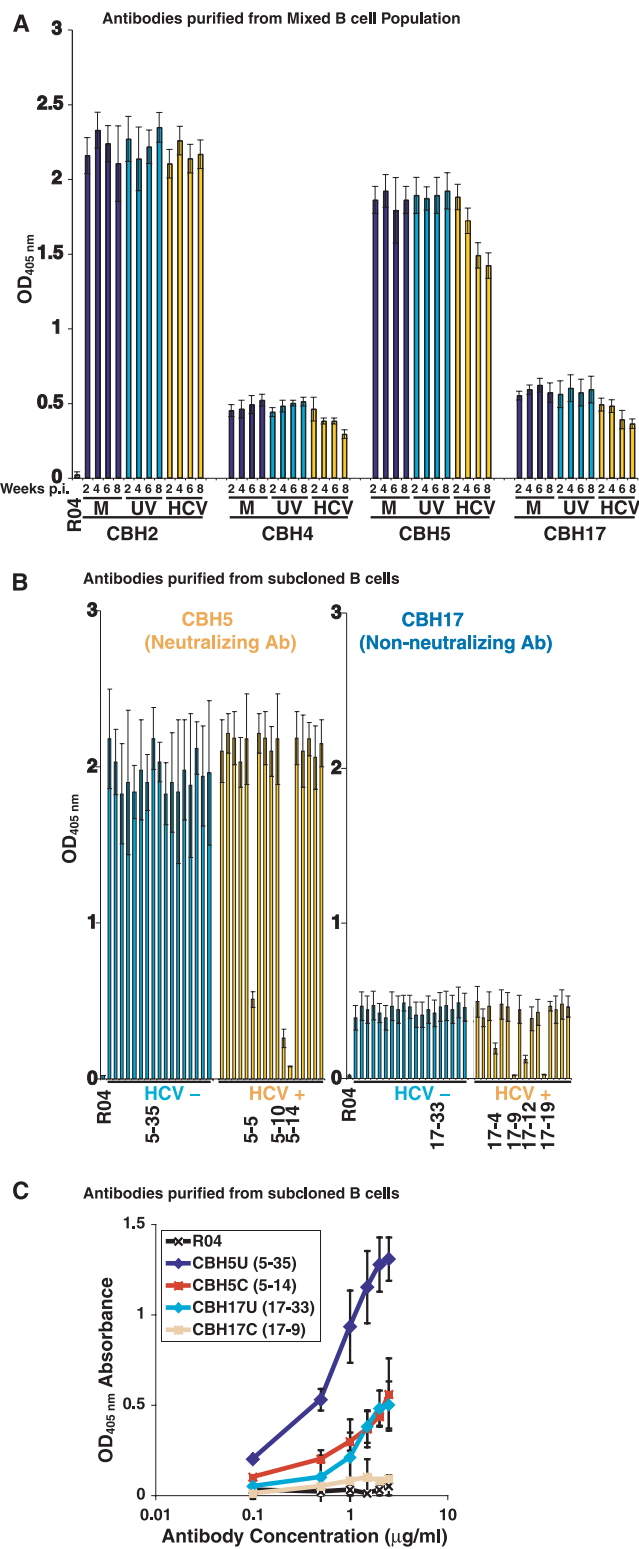


FIG. 3. E2-binding affinity of antibodies. (A) Antibodies secreted from pools of hybridomas collected at various time points (weeks postinfection). The binding assay is described in Materials and Methods. Antibodies were purified and normalized for binding assay. The binding activity is shown as the optical density at 450 nm. The same amounts of antibodies were used for ELISAs. M, mock infected; UV, infected with UV-irradiated virus; HCV, infected with HCV. (B) E2-binding activity of antibodies secreted from individual subclones of

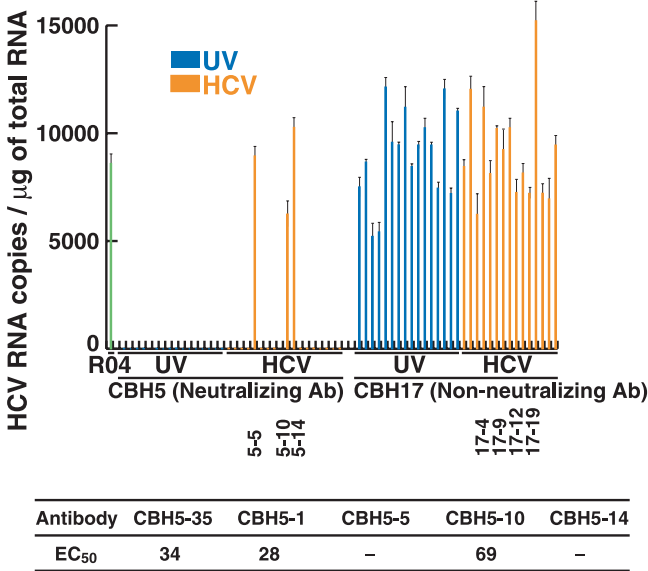


FIG. 4. Neutralizing activity of anti-E2 antibodies produced from individual subclones. SB-HCV was preincubated with antibodies produced from individual subclones and then used to infect naive Raji cells. Antibodies were purified from subcloned hybridomas and normalized for neutralization studies. HCV RNA was detected from infected Raji cells at 28 days after infection. The clone numbers are the same as in Fig. 2. The 50% effective concentration (EC₅₀) was calculated from the results obtained from different concentrations of antibodies, which were preincubated with HCV supernatant for infection. The EC₅₀ values for CBH5-5 and CBH5-14 are not shown since no neutralization activity was found in the neutralization assays.

hypermutation of immunoglobulin genes reduces the CDC activity.

DISCUSSION

This study presented evidence that HCV infection of antibody-producing B cells can result in the loss of binding affinity, virus-neutralizing activity and CDC of the antibodies produced by these cells because of the hypermutation of the immunoglobulin gene. Thus, the immunological (virus neutralization and CDC) functions of these antibodies are expected to decline during the course of viral infection. These findings may explain the puzzling observations that HCV persists in the sera of most HCV patients (1, 2, 44, 62). In the present study, we examined only the hybridoma clones producing E2- and E1-specific antibodies. Conceivably, HCV can infect B cells producing antibodies targeting other viral proteins and pro-

hybridomas (CBH5 and CBH17). The clone numbers are the same as in Fig. 2. Antibodies were purified from subcloned hybridomas and normalized for binding assay. (C) Antibodies secreted from individual subclones of infected (suffix C) or uninfected (suffix U) hybridomas were serially diluted and added to E2₆₆₁ (a carboxy-terminally truncated E2 without the transmembrane domain)-coated wells, and bound immunoglobulin was determined by ELISA. Three independent test results were averaged and calculated to determine the standard deviation.

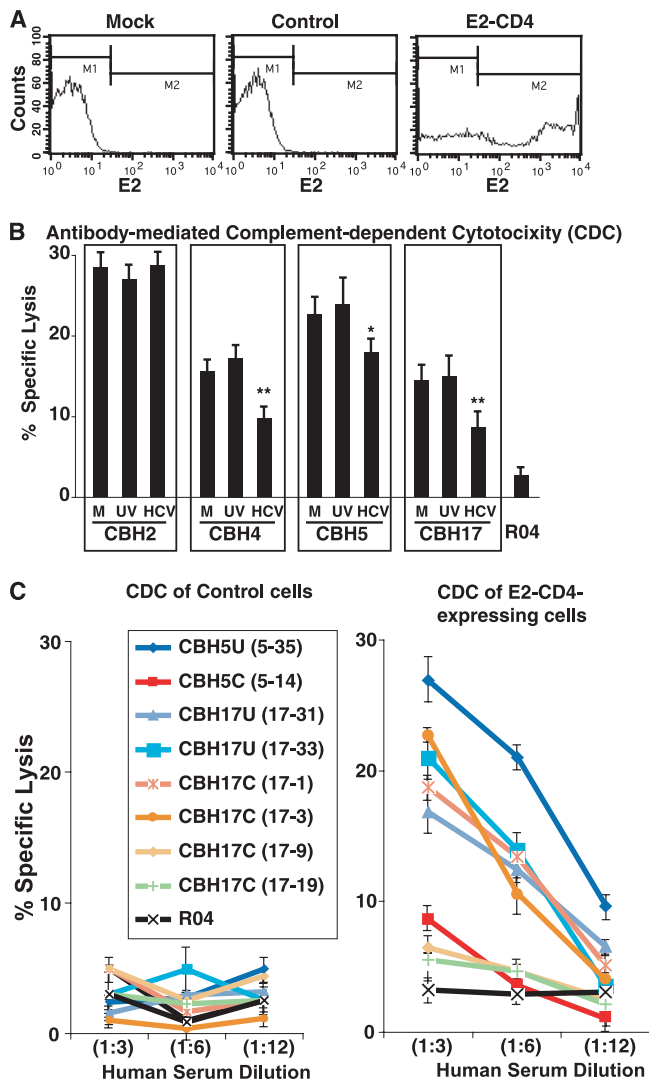


FIG. 5. CDC with the antibodies secreted from HCV-infected hybridomas. The assay protocol is described in Materials and Methods. (A) Surface expression of E2-CD4 in Huh7 cells infected with the recombinant vaccinia virus was determined by FACS using anti-E2 antibody. (B) CDC of pooled hybridomas. M, mock infected; UV, hybridomas infected with UV-irradiated virus. **, $P < 0.03$; *, $P < 0.05$. (C) CDC of individual subclones of infected (suffix C) or uninfected (suffix U) hybridomas. The clone numbers in parenthesis are the same as in Fig. 2. U, uninfected; C, HCV infected. The left panel shows results obtained with CDC using control Huh7 cells; right panel shows results obtained with E2-CD4-expressing Huh7 cells.

duce comparable effects on the immunological functions of those antibodies. Indeed, we have isolated a B-cell lymphoma cell line (SB cells), which is infected with HCV in vivo (57) and produces HCV NS3-specific IgM antibody (unpublished observation), indicating that HCV infection of antibody-producing B cells indeed occurred in vivo. This mechanism represents a novel mechanism for viral escape from immunosurveillance. It predicts that the neutralizing activity and CDC activities of HCV-specific antibodies will be lower and less specific in chronic hepatitis C patients (47). We have previously shown that the mutagenic effects of HCV on the Ig gene are induced by the binding of viral E2 protein to CD81, which is a core-

ceptor for B-cell receptor. Conceivably, other viruses (e.g., EBV) may also infect immune cells and cause hypermutation of Ig gene, since EBV protein gp350/220 interacts with CD21 (16, 48), which is also a coreceptor of B-cell receptor, and affects the expression of AID, which is responsible for Ig hypermutation (3, 61).

Ig hypermutation is usually considered to be a mechanism to select for an antibody of high affinity at the beginning of the immune response to virus infection. However, what we demonstrated here was that if the antibody-producing cells are infected with HCV, the quality of antibody may decline. It is not clear what selection forces are in operation here, since we found that most of the mutations were clustered in the CDR3 regions, which result in the loss of antigen-binding affinity. Some clones have frameshift mutations or deletions, which resulted in complete loss of antibody production. The latter clones were not further characterized in the present study. Thus, overall, there appeared to be a strong tendency for B cells to lose the virus-inactivating functions of their antibody. This selection force may also operate in vivo.

B cells that have undergone SHM leading to the expression of antibodies with increased affinity are positively selected in the light zone of the germinal center for continued proliferation and differentiation into memory B cells and plasma cells, whereas B cells with decreased affinity are selected against and die of apoptosis or anergy (10, 19, 53, 58). Thus, it is generally thought that normal B cells that undergo mutations that lead to decreased affinity and decreased neutralizing capacity would be lost during antigen selection, while those with higher affinity would come to dominate the response (30). If this applies to the response to HCV, in the course of the in vivo response, weaker B cells, such as the lower-affinity subclones of CBH5 hybridoma, would not be positively selected and would be lost, while the circulating antibodies on average would come from B cells that are making higher-affinity antibodies and undergo affinity maturation, leading to greater protection. Furthermore, in the case of HCV, B cells, such as CBH2 hybridoma, that are making higher-affinity and more effective neutralizing antibodies could not be infected and would not have their antibodies mutated to produce poorer antibodies. In fact, CBH2-like B cells would have an extra advantage and come to dominate the response, adding more B cells and making the most effective neutralizing antibodies to those producing higher affinity antibodies that are positively selected, resulting in an even greater dominance of neutralizing antibodies and a more protective response. However, it is possible that the induction of AID by HCV leads to the proliferation of B cells that are making antibodies that are less effective in eliminating the virus. It is also possible that the HCV-induced AID production and SHM contribute to malignant transformation of nongerminal center B cells outside of the germinal center, thus eliminating the positive selection for higher-affinity clones that goes on in the so-called "crucible" of the germinal center. The reported induction of AID and SHM in nongerminal center B cells, to some degree, would support this idea (67). In addition, there is the possibility that antibodies that lose their neutralizing capacity become Dengue-like non-neutralizing but enhancing antibodies and that these antibodies overcome or compete with the neutralizing antibodies. These possibilities would cause the decline of the protective power of anti-HCV

antibodies. The hybridomas used in the present study, however, were made from EBV-transformed human B cells fused to a mouse-human cell line. Therefore, we need to be careful to relate hybridoma studies to normal human B cells, since they have considerably different characteristics. We found that V-region mutations exist prior to HCV infection and that this may be due to low levels of AID (Fig. 2). More studies are required to reach a general conclusion.

At first glance, it seemed surprising that CBH5 cells could be infected with HCV, since CBH5 antibody can neutralize HCV. However, in our protocol, the hybridomas were infected with concentrated virus preparations; also, there was very little antibody in the culture supernatant to neutralize virus inoculums during *in vitro* virus infection, although HCV-like particles induce virus-specific humoral and cellular immune responses in mice with (51) or without (33) adjuvants or in a nonhuman primate model, such as the baboon (27) and chimpanzee (13). The corollary of this finding is that the expression of surface Ig did not prevent virus infection. Even if virus infection and replication did not actually occur in these hybridoma cells, the binding of E2 protein to CD81 of these cells should be sufficient to trigger the V_H hypermutation. It is also notable that the recombinant E2 alone could trigger V_H hypermutation, and yet the UV-inactivated HCV could not do the same. This could be explained by the low concentration of E2 on the surface of inactivated virus particles.

The clinical significance of B-cell infection by HCV has yet to be established. Compelling clinical data have shown that HCV causes extrahepatic infections (22, 43). Particularly, the prevalence of B-cell anomalies, which are responsive to interferon treatment (23, 34, 70), in hepatitis C patients in some geographical regions indicated strongly that HCV infects B cells and causes B-cell pathology (9, 18, 66). The SB strain of HCV used in the present study was derived directly from a B-cell lymphoma, which produces a monoclonal antibody specifically directed against HCV NS3 antibody, thus establishing unequivocally that this strain infects antibody-producing B cells *in vivo* (57). Furthermore, we have shown that this HCV strain preferentially infects lymphocytes (57) and that this lymphotropism is conferred by the viral envelope proteins (unpublished data). Therefore, HCV can infect B cells in at least some hepatitis C patients. It will be interesting to examine whether patients infected with lymphotropic HCV strains have a different clinical presentation from those patients infected with hepatotropic strains (17, 65). Although there is no global immune suppression during HCV infection, humoral antibodies in HCV patients were found to have only weak neutralizing activities (63), and the virus-neutralizing activities of antibodies are different between the acute and chronic phases of HCV infection (54, 63). How much of this difference is attributed to HCV infection of B cells with resultant Ig hypermutation remains to be investigated. It is significant to note that the SB strain of HCV can also infect T cells, particularly naive helper T cells (31). Thus, conceivably, T-cell functions may also be affected by HCV infection. It has been shown that helper T-cell functions are deficient in chronic hepatitis C patients, while those functions are robust in patients with self-limiting acute HCV infection (4). The binding of HCV to B cells (e.g., via CD81) could induce Ig hypermutation (38), which is physiologically significant.

We also demonstrated here that HCV-specific antibodies can induce CDC activity. Therefore, even non-neutralizing anti-E2 antibodies may play a significant protective or pathogenic role in HCV infection. This is a new aspect of viral immune response that has not been addressed in clinical studies of hepatitis C thus far. HCV E1 and E2 proteins are retained in the endoplasmic reticulum or cis-Golgi compartment (7, 12, 45) and exosomes (42). However, E1 and E2 are also detected on the surface of transiently transfected HEK 293T and Huh7 cells (11). Surface-localized E1E2 heterodimers are presented exclusively as noncovalently associated complexes and were incorporated into E1E2-pseudotyped human immunodeficiency virus type 1 particles that were competent for entry into Huh7 cells (11). In the virus-infected cells, viral envelope proteins may at least be transiently expressed on the cell surface, especially during entry (membrane fusion step) or release (budding step) in acute-phase infection. Transient exposure of surface E2 on infected cells may be sufficient to trigger CDC response. It will be interesting to examine how much CDC contributes to the immune response to HCV infection.

In conclusion, HCV infection of, or binding to, B cells induces hypermutation of immunoglobulin gene and inactivates neutralizing and CDC activity of antibodies produced by these cells. This model mechanism may contribute to viral escape from immunosurveillance, thereby helping to establish persistent infection.

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